IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: LITTLE et al.

Serial No.: 08/786,988

Filed: January 23, 1997

For: SYSTEMS AND METHODS FOR PREPARING AND ANALYZING LOW VOLUME ANALYTE ARRAY ELEMENTS

Art Unit: 1743

Examiner: Le, L.

ATTACHMENT TO RESPONSE

DECLARATION PURSUANT TO 37 C.F.R. §1.132 of Dr. Hubert Köster, with attached exhibit, Little *et al.* (1997) Anal. Chem. 69:4540-4546.

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DECLARATION PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, HUBERT KÖSTER, declare as follows:

1. I am a co-inventor of and familiar with the subject matter of the above-captioned application, which was filed on January 23, 1997.

2. I received a Ph.D. in chemistry from Hamburg University, Germany, and did post-graduate research work at the Max Planck Institute for Experimental Medicine in Göttingen, Germany. I was appointed professor of organic chemistry and biochemistry in 1982 at Hamburg University. I hold more than 20 patents and I have authored more than 110 publications.

3. I am a founder of several biotechnology companies, including Sequenom, Inc. I am presently its President and Chief Executive Officer.

4. I have reviewed the Office Action, mailed March 17, 1999, rejecting the claims in the above-captioned application over Tisone, U.S. Patent No. 5,743,960 ("Tisone") in view of Patterson, U.S. Patent No. 5,869,240 ("Patterson"). Neither reference teaches the results achieved when using substrates with spots resulting from delivery of small nanoliter volumes for mass spectrometry. As described below, use of such substrates for mass spectrometric analyses provide highly reproducible arrays of DNA samples for MALDI analysis. Such arrays are critical for performing high-throughput DNA diagnostics based on mass spectrometry. The methods and systems in this application provide high sample-to-sample uniformity and sample spots, which

can be entirely covered by the laser irradiation profile, thus eliminating difficulties associated with nonuniform analyte incorporation. The sample-to-sample uniformity that results when using substrates with small sample spots permits high spectrum reproducibility and high speed of spectrum acquisition. These improvements permit mass spectrometric MALDI analysis of analytes such as oligonucleotides, nucleic acids, peptides or proteins to be used high throughput applications.

5. My co-workers and I have prepared and analyzed arrays of synthetic oligonucleotides and DNA diagnostic products dispensed by a piezoelectric pipet as described in the above-captioned application. Results of such analyses have shown that sample delivery via small droplet size and the resulting small spots are advantageous for mass spectrometric analysis by providing shortened spectrum acquisition time, increased detection sensitivity, and greater reproducibility. These features permit incorporation of mass spectrometric analysis into high throughput automated protocols. Exemplary results are described below and also in the attached publication Little et al., *Anal. Chem.*, 69:4540-4546 (1997) (Exhibit A). I am a coauthor of this article, which presents results incorporated herein.

Experiments and Results

6. For sample dispensing, an Autodrop system from Microdrop GmbH (Norderstedt, Germany) was used (Exhibit A at page 4541, right column, first paragraph). The autodrop system from Microdrop GmbH includes the following components: 1) a piezoelectric element driver that sends a pulsed signal to piezoelectric element surrounding a silanized glass capillary (1.0 mm o.d., 70 μm i.d.) that holds the solution to be dispensed; 2) a pressure transducer to fill (by 10 mbar negative pressure) or empty (by 10 mbar positive pressure) the glass capillary; 3) a robotic *xyz* stage to maneuver the capillary for aspirating, dispensing, and capillary cleaning; 4) a stroboscopic light, which is pulsed at the frequency of the piezoelectric element to enabling viewing of "suspended" droplets; 5) separate stages for source (microplate) and sample (Si chip) targets; 6) a camera affixed to the arm holding the capillary, used to view the process of

dispensing into wells of the silicon chips; and 7) a Windows-based data station to control the pressure unit, *xyz* robot, and piezoelectric driver.

Typical pulse conditions for dispensing aqueous solutions were as follows: 200 Hz cycling of 60 V, 40 μs pulses of the piezoelectric element, with -10 mbar holding pressure on the active or inactive capillary to avoid dropping. Using these settings, the volume of a single droplet, determined by comparing the diameter of droplets illuminated with the stroboscopic camera to that of the capillary as viewed on a video screen, was about 300 pL/droplet. For dispensing nanoliter quantities, multiple 300 pL droplets were dispensed serially at the cycling rate of 200 Hz; each array element typically contained 15-20 droplets/element (4.5-6 nL/element total) (Exhibit A at page 4541, right column, second paragraph).

7. An array of samples containing 3-HPA, was prepared using the above subnanoliter dispensing means to dispense droplets. The resulting array included 100 μm diameter spots containing needle-shaped 5-25 μm micro crystals (Exhibit A at page 4543, Figure 1). It was found that this overall sample spot size approximates that of the focused laser spot, such that all crystals within the sample can be analyzed without adjusting the laser positioning once the sample spot is covered. The array of samples generated in this manner contains elements with highly homogenous crystals approximately centered within each individual well of the chip. The spot-to-spot variability between crystal size, depth, and general appearance was observed to be minimal relative to that typically observed with 3-HPA samples prepared by conventional pipetting.

8. The dispensed samples were analyzed by the time-of-flight mass spectrometry designed for such analysis (Exhibit A at page 4542, left column, fourth paragraph). MALDI-TOF (19 Kv extraction voltage, 500 ns pulse delay) spectra collected from each of the 100 wells using the semiautomated scanning mode of the x-y stage are shown in Figure 2 of Exhibit A (Exhibit A at page 4543). This data demonstrate distinct benefits from the uniform spot-to-spot

crystal characteristics and from the small size of the spots, such as high reproducibility among the mass spectra corresponding to each sample.

9. Acquisition of the mass spectrometry spectrum set of the subnanoliter-dispensed sample array was rapid. In fact, manually saving each individual spectrum was the major bottleneck in speed. A scan in which the elements were analyzed until intense signal was observed but the spectra were not stored required only 300 seconds, or 18 spectra/min. (Exhibit A at page 4543, left column, first full paragraph).

10. The detection limit for MALDI analysis of DNA from the miniaturized samples was examined for a synthetic 36-mer (Exhibit A at page 4544, the paragraph adjourning left and right columns). Five concentrations were tested: 4.1 fmol (910 nM), 2.0 fmol (455 nM), 0.8 fmol (180 nM), 0.4 fmol (90 nM) and 0.2 fmol (45 nM). Representative spectra were shown in Figure 3 of Exhibit A ((Exhibit A at page 4544). For elements containing 0.4 fmol or more than 0.4 fmol, spectrum acquisition was essentially immediate upon irradiation. Acquisition from half of the spots containing 0.2 fmol resulted in no discernible signal, but the other five spots yielded low-intensity signals from which molecular mass values could be obtained. These spectra represent nearly an order-of-magnitude higher sensitivity than that previously reported for MALDI of oligonucleotides in this size range.

11. In contrast, when arrays with larger spots were used there was dramatic variability of analyte incorporation and ion yields from different regions of crystals obtained from conventional large-volume (*e.g.*, 300 nL) preparations. Typically it is necessary to manually search within the 1-2 mm diameter spots for regions from which intense signals can be obtained. The difficulty in finding satisfactory crystals regions increases dramatically with decreasing analyte concentrations.

With the methods of the application, such search is not necessary for miniaturized samples because the dimensions of the laser spot are approximately equal to those of the total sample size. Thus, compared to arrays produced

using conventional sample dispensing methods, spectrum acquisition is far more routine and amenable to automation with the miniaturized samples.

12. Miniaturized sample dispensing methods were used in dispensing biological samples generated in a temperature-cycled primer oligo base extension (PROBE) reaction (described in co-pending U.S. Patent Application Serial Nos. 08/617,256 and 08/744,481). Generally, a PROBE reaction includes the steps of: 1) obtaining, and preferably amplifying, a nucleic acid containing the locus of interest, 2) separating and/or purifying one strand of the nucleic acid by binding it to a solid support; 3) annealing a primer, which is complementary to a region adjacent to the locus of interest, to the immobilized strand; 4) adding appropriate polymerase, elongating trinucleotides (*e.g.*, dNTPs) and terminating trinucleotides (*e.g.*, ddNTPs); and 5) allowing the polymerase to incorporate nucleotides via template-directed synthesis onto the 3'-end of the primer so that it is extended through the locus of interest. The number of bases incorporated and, hence, the molecular mass of the extension products give the identity of the base(s) at the locus of interest. Because of the relatively small differences in molecular weights that are detected by this method, high reproducibility is essential for the method to be practical and to perform reliably.

In one example, a region of the apolipoprotein B gene was examined to determine the identity of the codon 3500 mutation associated with abnormal cholesterol transport and metabolism (Exhibit A at page 4545, left column, second full paragraph). The MALDI spectra of this analysis is shown in Figure 5 of Exhibit A (Exhibit A at page 4545), which indicates that the sensitivity of MALDI from miniaturized samples is well above that needed for highly accurate routine mutation typing.

13. The preparation of highly reproducible arrays of DNA samples for MALDI analysis as described above is a critical aspect in making high-throughput DNA diagnostics based on mass spectrometry routine. The methods and systems in this application provide high sample-to-sample uniformity and sample spots, which can be entirely covered by the laser irradiation profile, thus

eliminating difficulties associated with nonuniform analyte incorporation. This results in high spectrum reproducibility and high speed of spectrum acquisition. These improvements permit mass spectrometric MALDI analysis of analytes such as oligonucleotides, nucleic acids, peptides or proteins to be used high throughput applications.

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

HUBERT KÖSTER

Date: 2/5/00

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